Cytoskeletal dynamics and cell signaling during planar polarity establishment in the *Drosophila* embryonic denticle

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Summary

Many epithelial cells are polarized along the plane of the epithelium, a property termed planar cell polarity. The *Drosophila* wing and eye imaginal discs are the premier models of this process. Many proteins required for polarity establishment and its translation into cytoskeletal polarity were identified from studies of those tissues. More recently, several vertebrate tissues have been shown to exhibit planar cell polarity. Striking similarities and differences have been observed when different tissues exhibiting planar cell polarity are compared. Here we describe a new tissue exhibiting planar cell polarity – the denticles, hair-like projections of the *Drosophila* embryonic epidermis. We describe in real time the changes in the actin cytoskeleton that underlie denticle development, and compare this with the localization of microtubules, revealing new aspects of cytoskeletal dynamics that may have more general applicability. We present an initial characterization of the localization of several actin regulators during denticle development. We find that several core planar cell polarity proteins are asymmetrically localized during the process. Finally, we define roles for the canonical Wingless and Hedgehog pathways and for core planar cell polarity proteins in denticle polarity.

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Introduction

One hallmark of living cells is that they possess polarity, with plasma membrane regions differentiated from one another in protein content and function (Nelson, 2003). Epithelial apical-basal polarity is a well-studied example. Many epithelial cells possess a second axis of polarity perpendicular to the apical-basal axis. This polarization along the plane of the epithelial sheet, frequently along body axes, such as anterior-posterior, dorsal-ventral or proximal-distal, is termed planar cell polarity (PCP) (reviewed in Axelrod and McNeill, 2002; Eaton, 2003; Fanto and McNeill, 2004).

PCP was originally characterized and molecules required for its establishment identified in *Drosophila*. The developing wing provides an excellent model of this phenomenon. The wing imaginal disc is a simple epithelial sheet whose cells have their cytoskeletons polarized along the proximal-distal axis, such that each wing cell ultimately develops an actin and microtubule-based cellular projection called a wing hair at its distal-most vertex (Wong and Adler, 1993). To do so, the cell must do two things: (1) determine an axis of polarity; and (2) regulate cytoskeletal assembly in response to this.

The first clues to the mechanisms underlying PCP emerged from work pioneered by Adler and others, who identified genes essential for wing hair polarity. These 'core' polarity genes include *frizzled* (*fz*), *dishevelled* (*dsh*), *flamingo/starry night* (*fmi*), *Van Gogh/strabismus* (*stbm*) and *prickle* (*pk*) (reviewed in Adler, 2002). They are required for cells to establish polarity, with the cytoskeletal events of wing hair development as downstream consequences, and also regulate PCP in other adult epidermal structures, determining ommatidial orientation in the eye and bristle polarity in the abdomen.

Planar cell polarity and the machinery involved in its generation are broadly distributed in animals. During vertebrate gastrulation and neurulation (convergent extension), cells become polarized with respect to their migratory properties, and Fz and Dsh homologs regulate this process (Djiane et al., 2000; Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). In mice, stereociliary bundles of the sensory hair cells of the inner ear exhibit PCP, and mutations in Fmi (Curtin et al., 2003) and Van Gogh/Strabismus homologs (Montcouquiol et al., 2003) disrupt this. Fz6 orients the hairs in mouse fur (Guo et al., 2004).

Fz proteins are receptors for Wnt ligands (Bhanot et al., 1996), cell-cell signals shaping cell fates in all animals (reviewed in Logan and Nusse, 2004). The involvement of Fz in PCP raised the possibility that Wnt protein gradients across polarized tissues might orient cells. Consistent with this

hypothesis, zebrafish Wnt11 regulates convergent extension (Heisenberg et al., 2000) and Wnt7A may regulate planar polarization in the inner ear (Dabdoub et al., 2003). Given this, it seemed plausible that a gradient of Wingless (Wg; a fly Wnt) across the pupal wing or eye might be the signal that polarized cells. However, in these tissues PCP does not appear to require Wg or the downstream components of its canonical signaling pathway like Armadillo (Arm) (Boutros et al., 1998; Boutros et al., 2000; Rulifson et al., 2000; Struhl et al., 1997; Wehrli and Tomlinson, 1998; Yang et al., 2002). Of course, without loss-of-function mutations in all *Drosophila* Wnt proteins, one cannot rule out all roles for Wnt signaling.

In the eye, Fz activity is modulated by a novel mechanism involving gradients of two transmembrane proteins, Four-jointed (Fj) and the cadherin-superfamily member Dachsous (Ds), rather than by a Wnt gradient. Fj and Ds are thought to act through the cadherin-superfamily proteins Fat and Fmi to influence Fz activity by an unknown mechanism (Simon, 2004; Yang et al., 2002). These proteins also regulate PCP in other tissues, but the details differ. Ds and Fj form opposing gradients in the abdomen, but cells interpret this information differently in the anterior and posterior compartments (Casal et al., 2002). In developing wings, by contrast, while Ds and Fj are expressed in gradients (Ma et al., 2003; Zeidler et al., 2000) and ectopically altering these gradients alters polarity (Matakatsu and Blair, 2004), the gradients can be experimentally flattened without disrupting polarity (Simon, 2004). In addition, Fat, Ds and Fj regulate Wg expression and function during wing growth (Cho and Irvine, 2004; Rodriguez, 2004) – whether this has implications for PCP remains to be seen.

Once an axis of polarity is established, the cytoskeleton must be polarized in response to it. Elegant analysis of wing development revealed that each hexagonal epithelial cell initially forms an actin-rich condensation at its distal vertex, which is the precursor for the wing hair (Wong and Adler, 1993). Microtubules (MT) form the core of the wing hairs, and disruption of either actin or MTs disrupts their development (Eaton et al., 1996; Turner and Adler, 1998). Core polarity genes position the initial actin accumulation at the distal cell vertex – in the absence of any core component, actin accumulates and wing hairs initiate at the center of the cell's apical domain (Wong and Adler, 1993).

A subset of the genes affecting PCP act more directly in cytoskeletal regulation, including known regulators like RhoA and Rho-kinase, and less well-characterized or uncloned proteins like Tricorned, Inturned, Furry, or Multiple wing hairs (reviewed in Adler, 2002). However, the mechanism by which a polarizing signal is translated into changes in cytoskeletal organization remains unclear. The core polarity proteins also regulate PCP of sensory bristles. Much is known about subsequent regulation of actin assembly there, revealing roles for actin cross-linkers and other actin regulators (Guild et al., 2003; Hopmann and Miller, 2003; Tilney et al., 1998; Tilney et al., 2000b; Wahlstrom et al., 2001).

We identified an alternate model in which to examine the signaling and cytoskeletal events of *Drosophila* PCP: the embryonic denticles (Fig. 1A,B) (Martinez-Arias, 1993). Denticles are actin-based cell projections present on segmentally repeated subsets of ventral epidermal cells (Dickinson and Thatcher, 1997); in the abdomen the most posterior cell and the five most anterior cells of each segment make denticles (Martinez-Arias, 1993). The embryonic epidermis presents many advantages for studying the signaling and cytoskeletal events required for PCP. Its cells are large, easily visualized in live and fixed preparations, and the genetic circuitry underlying their development is well understood.

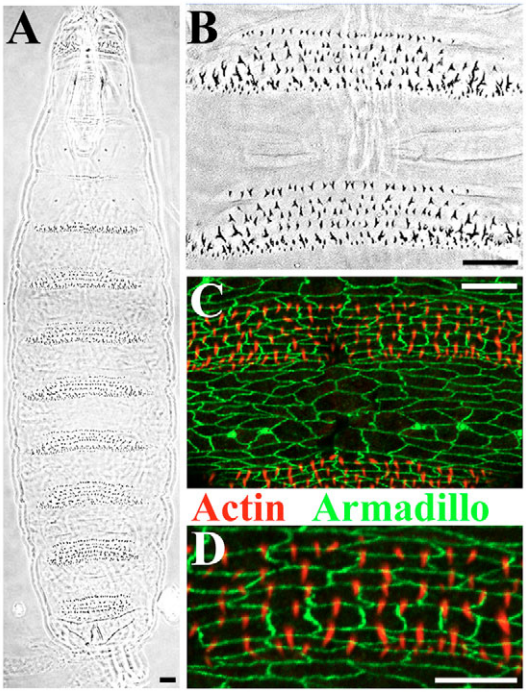


Fig. 1. Denticles. Embryos, anterior at top. Unless noted, embryos here and in other Figures are wild type. (A,B) Cuticles. Six rows of cells per segment produce cuticle-covered cell projections called denticles. (C,D) F-actin (red). Arm (green, outlining cells). Denticles are actin-based and initially point posteriorly. Scale bars, 15 μm.

Results

Cytoskeletal rearrangements during wild-type denticle development

Dickinson and Thatcher (Dickinson and Thatcher, 1997) established that denticles are actin-containing cell projections, and found that fully extended denticles all point posteriorly (Fig. 1C,D). Later in development, presumably after cuticle deposition, denticle rows 1 and 4 come to point anteriorly (Martinez-Arias, 1993) (Fig. 1B). We examined cytoskeletal events underlying denticle formation, using fluorescently labeled phalloidin and anti-actin antibodies to examine fixed samples (Fig. 2A-H), and examined F-actin dynamics using the F-actin-binding domain of *Drosophila* Moesin fused to green fluorescent protein, hereafter called Moesin-GFP (Fig. 2I-P) (see also supplementary material Movies 1a,b) (Edwards et al., 1997). Both approaches yielded similar pictures of the process.

Denticle formation occurs over ~2 hours during mid-embryogenesis. During early to mid-embryogenesis, cells are roughly columnar (Fig. 2A), with actin in a belt underlying the adherens junctions. ~1 hour before the first signs of denticle formation (at the onset of dorsal closure), ventral epidermal cells change shape, narrowing along the anterior-posterior axis and extending along the dorsal-ventral axis. Cells that will later

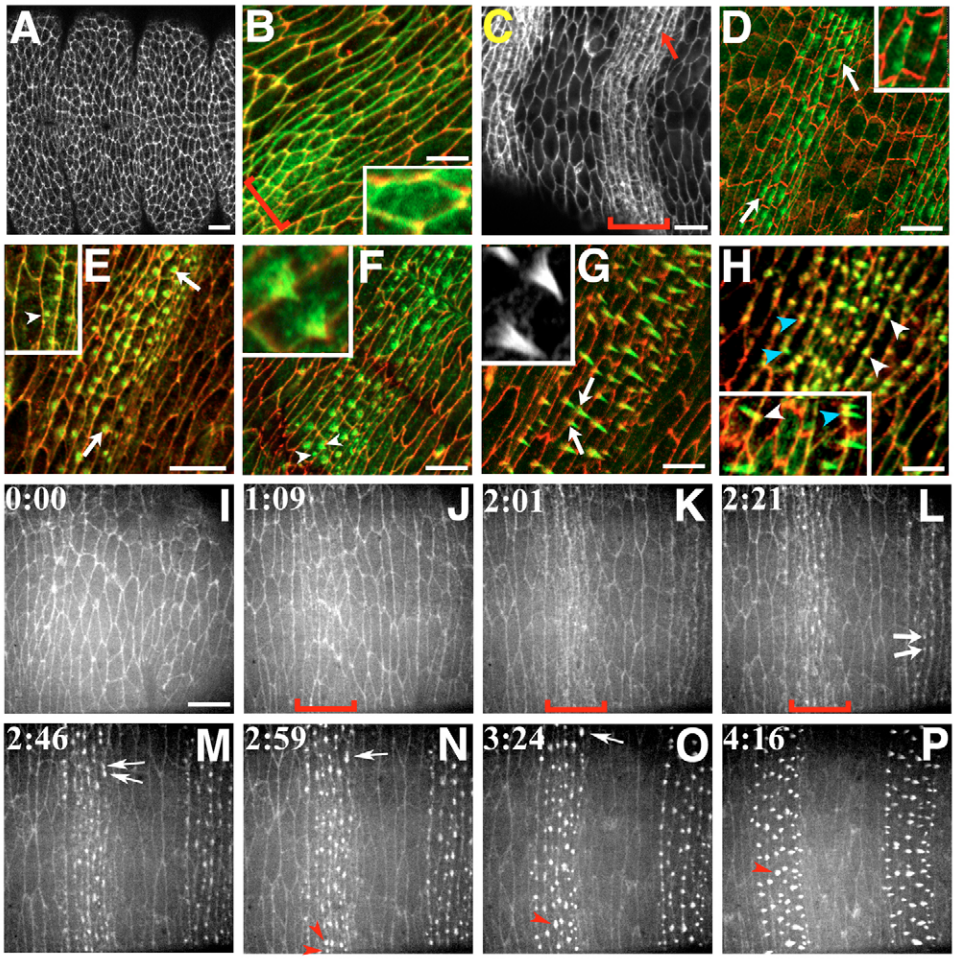


Fig. 2. Denticles develop from polarized actin accumulations. Embryos, anterior left or upper left, F-actin (green or single label; A-C,E-H=phalloidin; D=anti-actin) and P-Tyr (B,E-H) or Arm (D) to outline cells (red). Insets=close-ups. (A) Extended germband. (B,C) Cells of presumptive denticle belts accumulate apical actin (brackets, B inset), beginning laterally (B, bottom left). (C,D) Actin concentrates along posterior cell margins (arrows). Insets B,D are close-ups. (E) Actin condenses into foci along the posterior margin (arrows). Inset, rare error in denticle positioning in row 1 (arrowhead). (F,G) Stages in denticle elongation (arrows, arrowheads, multiple denticles/cell). (H) Dorsal hairs. Condensations form and hairs (inset) elongate at the posterior-margin (blue arrowheads) or anterior-margin (white arrowheads) in different cell rows. (I-P) Stills, movie of Moesin-GFP-expressing embryo. Times in hours:minutes. (I) Before denticle formation. (J,K) Actin accumulates apically in presumptive denticle belts (brackets). (L) Actin foci along the posterior margin (arrows). (M-O) Foci condense and brighten. White arrows, red arrowheads – foci merging. (P) Denticles begin elongation. Scale bars, 10 μm.

make denticles become narrower along the anterior-posterior axis than cells that will make naked cuticle (Fig. 2B,C,K brackets). Cells of presumptive denticle belts also begin to accumulate actin in a meshwork uniformly covering the apical surface, while cells that will make naked cuticle do not (Fig. 2B,C,K brackets, B inset). Apical actin accumulation and subsequent events in denticle development are initiated in lateral regions of each denticle belt and move medially (Fig. 2B, lateral to the lower left).

Within an hour of apical actin accumulation, polarity becomes apparent. The uniform meshwork of apical actin 'condenses' along the posterior margin of each cell (Fig. 2C,D, arrows). We often observed multiple actin condensations (Fig. 2C,D arrows; D inset). Ultimately, most cells of the denticle belt contain a single large condensation (Fig. 2E, arrows). Our time-lapse movies of Moesin-GFP (Fig. 2I-P) revealed that at least some of these form when smaller condensations merge (Fig. 2L-P, arrows and arrowheads). Different denticle rows differ in average denticle number/cell, ranging from ~1.0 (row 5) to 2.1 (row 1; data not shown). The fact that some cells retain >1 condensation (Fig. 2F, arrowheads) may account for the production of multiple denticles by some cells (Fig. 2G, arrows). While there is a strong posterior bias, occasional mistakes are made; surprisingly, these are almost totally confined to rows one and two. During condensation, occasional denticles are not tightly associated with the posterior margin (data not shown) – this usually resolves during denticle elongation, but in ~10% of the belts a mispositioned denticle can be detected (Fig. 2E inset, arrowhead)

Actin condensations sharpen and brighten over time, remaining tightly associated with the posterior cell margin (Fig. 2O). Around 100 minutes after the first actin accumulation, denticles began to elongate posteriorly over neighboring cells (Fig. 2F,F inset, P), gradually assuming their final size (Fig. 2G,G inset). As Dickinson and Thatcher (Dickinson and Thatcher, 1997) observed, all denticles initially elongate posteriorly (Fig. 2G). Anterior bending of rows 1 and 4 and the development of a curved shape (Fig. 1B) presumably occur later. Cuticle deposition (preventing examination of fixed tissue) and muscle movements (making filming live embryos problematic) make these events difficult to visualize. We also examined dorsal hair formation. Although we did not study this in detail, the process seems similar, with initial actin condensations (Fig. 2H) and later elongation (Fig. 2H, inset). Like denticles, dorsal hairs are polarized, but unlike denticles, in different rows condensations initiate at and dorsal hairs project from either the anterior (Fig. 2H, white arrowheads) or posterior (Fig. 2H, blue arrowheads) cell margins.

In examining actin in fixed tissue, we used antibodies to phosphotyrosine (P-Tyr) to outline cells. P-Tyr co-localizes with actin in condensations, where it often marked a slightly larger area than that occupied by actin (Fig. 3A, arrows), and

in extending denticles, where it was weaker in the denticle tip (Fig. 3B, blue arrow) but enriched near the base (Fig. 3B, white arrow). The identities of the Tyr-phosphorylated proteins are not known.

To get a clearer picture of actin organization, we combined confocal microscopy and deconvolution; this technique sharpens the image by removing out-of-focus light. Initial actin condensations do not have obvious substructure (Fig. 3E1). Mature actin condensations sometimes appeared 'donut-shaped', with less actin in the center (Fig. 3E2). As denticles elongated, this 'hollow center' became more apparent, with denticles appearing U- or V-shaped (Fig. 3E3). Next, the actin took on a 'shovel-shape' with the open side of the 'shovel' on the apical side of the developing denticle (Fig. 3E4). The deconvolved image stacks also revealed that all actin structures observed, from the initial apical accumulation, occur at or very near the apical surface of the cell (data not shown).

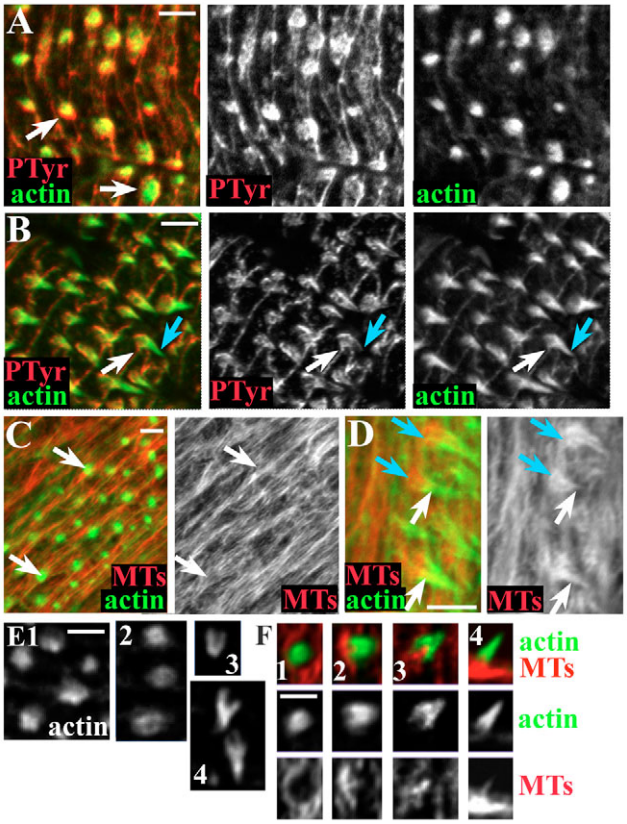


Fig. 3. MT and P-Tyr localization during denticle development. (A-D) Embryos, anterior left (A,D) or upper left (B,C). F-actin (phalloidin, green), P-Tyr (A,B red) or MTs (C,D red). (A) P-Tyr (red) accumulates with and around actin (green) in condensations (arrows). (B) P-Tyr (red) is enriched at denticle base (white arrow) but less prominent at the tip (blue arrow). (C) MTs (red) form circumferential arrays without enrichment near condensations (green, arrows). (D) MTs enriched at the denticle base (blue arrows) and in the core (white arrows) during elongation. (E,F) Deconvolved confocal images. (E) F-Actin (phalloidin). (E1) Early condensations. (E2) Late condensations with 'donut holes'. (E3,E4) U- or V-shaped denticles. (F) MTs (red). F-actin (Phalloidin; green). (F1) MTs encircling a condensation. (F2,F3) MTs in the core of V-shaped denticles. (F4) Fully elongated denticle. MTs at base and in core. Scale bars, 5 μm.

In parallel, we examined microtubules (MTs), which play an important role in wing hair formation (Turner and Adler, 1998). Dickinson and Thatcher (Dickinson and Thatcher, 1997) reported that during denticle development, MTs are organized in circumferential arrays, with no association with nascent denticles. Our data support this through the condensation stage (Fig. 3C). However, as denticles elongate, MTs become enriched at the denticle base (Fig. 3D, blue arrows) and in the denticle core (Fig. 3D, white arrows). This was even clearer in deconvolved images (Fig. 3F), with MTs at the core initially visible when the denticles became U- or V-shaped (Fig. 3F2), and remaining in the core as denticles continue to elongate (Fig. 3F3,4). Thus, the core of maturing denticles appears to contain MTs.

The localization of cytoskeletal regulators

To identify actin regulators that may modulate denticle development, we localized three candidates: the formin Diaphanous (Dia), the Arp2/3 complex and Enabled (Ena). Dia regulates actin nucleation and extension and MT stability (reviewed in Zigmond, 2004). Fly Dia regulates actin organization before and during cellularization (Afshar et al., 2000), but its later localization was unknown. Before denticle initiation (late extended-germband), Dia localizes cortically in all cells (Fig. 4A). It becomes enriched in developing denticles (Fig. 4B, white arrowheads) but remains exclusively cortical in cells without denticles (Fig. 4B, blue arrowhead). In deconvolved images, Dia is cortical early in the process, with only traces in actin condensations (Fig. 4C,C′, white arrowhead). As denticles elongate, Dia localizes in the vicinity of denticle primordia (Fig. 4B′,D,D′, white arrowheads) and to denticles (Fig. 4E,E′), though in a more punctate fashion than actin.

The Arp2/3 complex nucleates actin filaments and creates branched filament networks (reviewed in Pollard and Borisy, 2003). In syncytial embryos, Arp3 is enriched in the vicinity of actin structures, but is not restricted to these locations (Stevenson et al., 2002). Its localization later in embryogenesis was not previously reported. During germband extension, we observed weak enrichment of Arp3 at cell borders, along with strong punctate cytoplasmic staining (data not shown). Later Arp3 is enriched in denticles. Deconvolution revealed that this began with weak enrichment around actin condensations (Fig. 5A,A′, blue arrowhead) and in denticles beginning to extend (Fig. 5B,B′, blue arrowheads), while Arp3 is strongly enriched in elongated denticles (Fig. 5C,C′, blue arrowhead)

Ena promotes actin filament elongation (Bear et al., 2002). It localizes to cell-cell junctions in epithelial cells (Baum and Perrimon, 2001; Grevengoed et al., 2001; Vasioukhin et al., 2000). In extended germband embryos, Ena is strongly enriched at 'tricellular junctions' where three cells meet. During early denticle development, Ena becomes more uniformly localized around the circumference of cells that will make denticles (Fig. 5D, brackets) while remaining predominantly at tricellular junctions in other cells (Fig. 5D,F-H, blue arrowheads). Ena is not strongly enriched in actin condensations (Fig. 5G,G′, yellow arrowhead). Ena becomes enriched in denticles as they elongate (Fig. 5H′,I′ yellow arrowheads) and enrichment is greatest near the end of elongation (Fig. 5F, bracket). In contrast to Dia, Ena is enriched at the denticle base (Fig. 5I,I′, inset, white arrowhead=denticle

in cross-section), though Ena also localizes to the denticle itself. Thus, in summary, three actin regulators are differentially enriched in denticles during their development.

APC2 and an APC2-GFP fusion protein co-localize with actin in denticles (Cliffe et al., 2004; McCartney et al., 1999). APC2 might influence denticle development as a cytoskeletal regulator that interacts with both MTs and actin, or as a regulator of Wg signaling. We thus characterized APC2 localization throughout denticle development, using immunofluorescence of fixed tissue (Fig. 6A,B) and an APC2-GFP fusion (Fig. 6C-H) (see also supplementary material Movie 2). Prior to denticle development, APC2, like actin, localizes to the apical junctions, outlining cells (McCartney et al., 1999; Yu et al., 1999). As denticle development initiates, APC2 becomes enriched in an apical meshwork covering cells of the presumptive denticle belt (Fig. 6C, brackets), reminiscent of actin. APC2-GFP recapitulates the behavior of Moesin-GFP (Fig. 2I-P) fairly precisely, becoming enriched along the posterior margin (Fig. 6E, arrows) and coalescing into condensations that sometimes merge (Fig. 6F-H, colored arrowheads). We also observed posterior enrichment and localization to condensations in fixed tissue (Fig. 6A,B, data not shown), although the anti-APC2 signal was more diffuse than that of actin or APC2-GFP. This difference may reflect a more diffuse distribution of endogenous APC2 than of APC2-GFP, or it may be a fixation artifact.

APC2 binds Arm and co-localizes with DE-cadherin at several stages of development (McCartney et al., 1999; McCartney et al., 2001; Yu et al., 1999). To determine whether these APC2 partners localize to denticles, we examined DE-cadherin (Fig. 7A) and Arm (Fig. 7B), as well as the septate junction protein Coracle (Fig. 6I), which plays a role in the parallel alignment of wing hairs (though not their polarity) (Venema et al., 2004). None of these proteins accumulate in developing denticles (Fig. 7A,B, white arrows, Fig. 6I). However, higher levels of cortical Arm are seen in cells that make denticles (Fig. 7B, brackets) and it is slightly enriched on dorsal-ventral cell margins (Fig. 7B,B′ blue arrowheads) relative to the anterior-posterior cell margins (Fig. 7B,B′, red arrows).

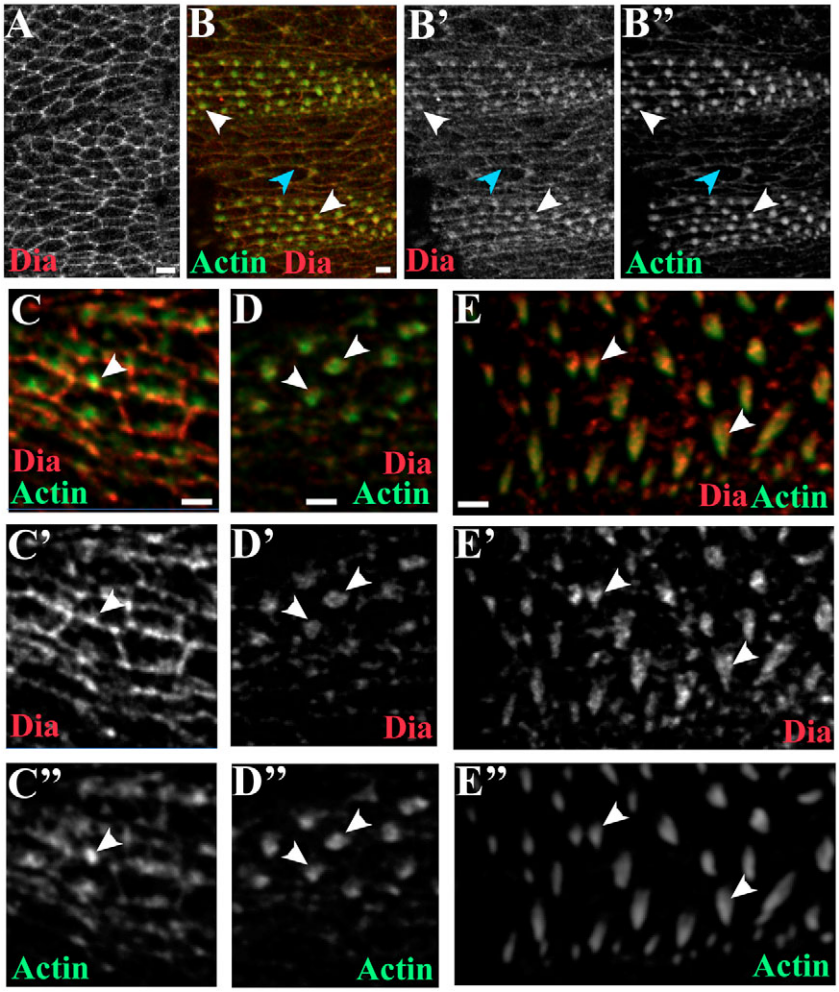


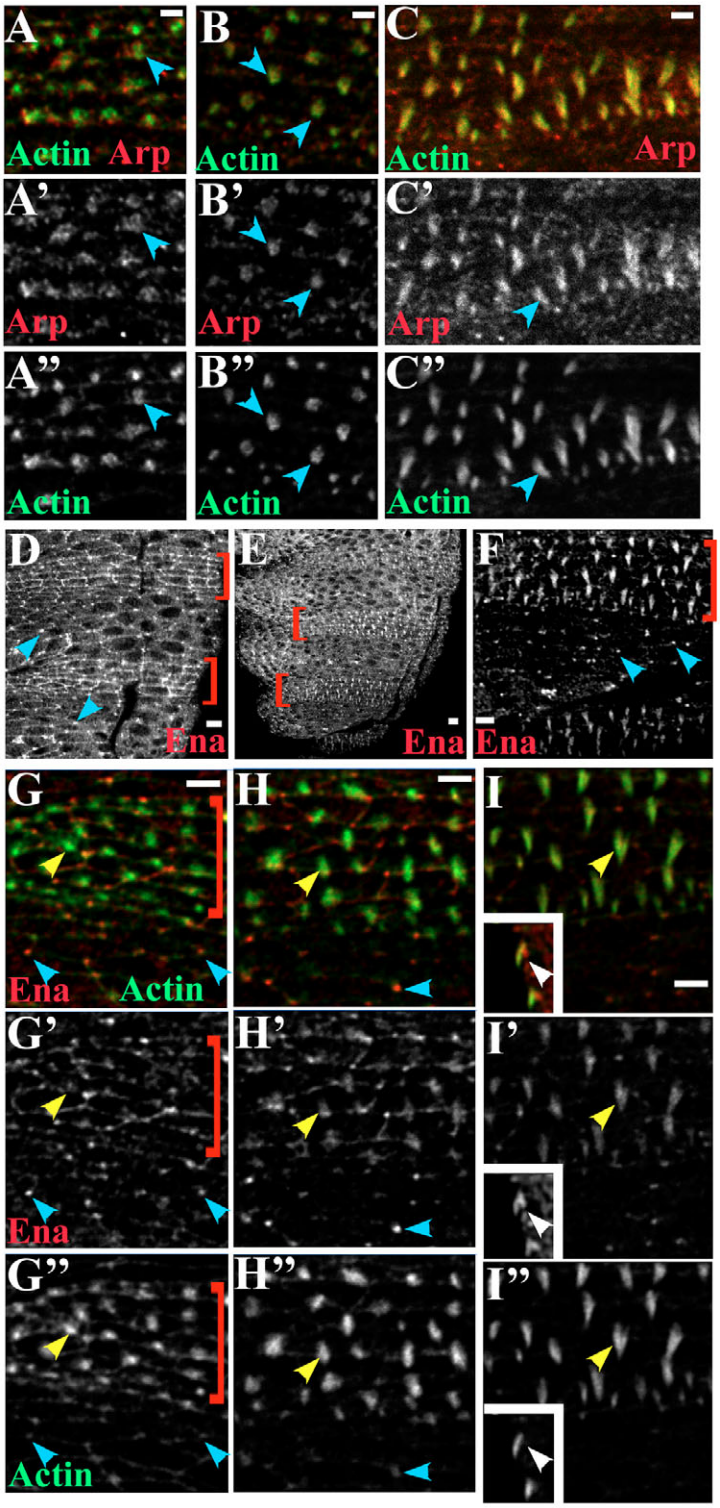
Fig. 4. Dia in denticles. Embryos, anterior to top. Dia (red), F-actin (phalloidin; green). (A) Before denticle development. (B) As denticles elongate, Dia becomes enriched in denticles (white arrowheads) while remaining cortical (blue arrowheads) in non-denticle-producing cells. (C-E) Deconvolved confocal images. (C) No substantial enrichment in early actin condensations (arrowhead). (D) Dia begins accumulating in elongating denticles (arrowheads). (E) Dia accumulates in a punctate fashion in elongated denticles (arrowheads). Scale bars, 5 μm.

Polarized accumulation of polarity proteins

To identify factors that might regulate denticle polarity, we examined the localization of several regulators of PCP in the wing. Fmi is one of several proteins required for polarity that localizes in a polarized fashion during wing hair development – Fmi accumulates at proximal and distal cell boundaries (Chae et al., 1999; Usui et al., 1999; Shimada et al., 2001). In the dorsal epidermis, Fmi accumulates preferentially along the anterior and posterior margins of cells migrating toward the dorsal midline (Kaltschmidt et al., 2002). In the ventral epidermis, Fmi expression is weak during the extended germband (data not shown), but it accumulates uniformly around the cortex before denticle formation is initiated (Fig. 7C). Fmi becomes polarized as denticles begin to form (Fig. 7D; 7E, Fmi is green; Arm is violet). In cells that make denticles, Fmi accumulates at higher levels along the anterior and/or posterior cell margins (Fig. 7E, red arrows) and lower levels along dorsal-ventral cell borders (Fig. 7D,E blue arrowheads).

We next examined Fz and Dsh localization using GFP-fusions – both are also polarized during wing hair development (Strutt, 2001; Axelrod, 2001). Fz-GFP (Fig. 7F,G) (see also supplementary material Movie 3) and Dsh-GFP (Fig. 7H-K) both were slightly polarized, with elevated accumulation on the anterior and/or posterior borders (Fig. 7F,I,J red arrows, arrowheads). Dsh-GFP accumulated in a fashion reminiscent

of actin, forming accumulations along the posterior margin and posterior condensations (Fig. 7J,K blue arrows). Fz-GFP was more generally enriched along the anterior/posterior margin. Before denticle development, we were surprised to see that Fz-GFP primarily accumulates in motile intracellular puncta we presume are vesicles (Fig. 7F, blue arrowheads) (see also supplementary material Movie 3) – only after cell elongation does it become predominantly localized to the cell surface. Dsh-GFP was also seen in presumptive vesicles (Fig. 7H, blue arrowheads), though it accumulated at the plasma membrane throughout.



Correct denticle polarization requires Wg and Hedgehog signaling

PCP in *Drosophila* requires Fz and Dsh but, unlike vertebrates, it does not appear to require either Wg or downstream components of the canonical signaling pathway, such as Arm (see Introduction). *Drosophila wg* is a segment polarity gene (Nüsslein-Volhard and Wieschaus, 1980), reflecting the fact that in cuticle preparations all ventral epidermal cells secrete denticles, but within each segment the denticles have mirror-image polarity. This phenotype suggested that Wg might play a role in denticle PCP. To determine whether changes in denticle polarity reflect alterations in cytoskeletal polarity, we examined denticle formation in embryos mutant for *wg* or components of its signaling pathway.

The first notable difference in the null mutant wg^{IG22} precedes denticle development: cells remain columnar rather than elongating along the dorsal-ventral axis (Fig. 8A). A similar failure of cell shape changes occurs dorsally during dorsal closure (McEwen et al., 2000), perhaps reflecting a broader role for Wg in cell polarization or cytoskeletal regulation. As previously observed, cell number is substantially reduced due to excess apoptosis (Pazdera et al., 1998). All surviving ventral epidermal cells produce denticles (Nüsslein-Volhard and Wieschaus, 1980).

Differences are apparent in *wg* mutants from the start: when denticle development initiates, all cells within the segment accumulate a meshwork of apical actin. This begins at the lateral segment margins (Fig. 8A, brackets), as in wild-type. Within each cell, actin then begins to condense (Fig. 8B,C, arrowheads), usually coalescing into a single condensation per cell (Fig. 8E). However, in contrast to wild type, actin condensations do not invariably form at posterior cell margins. Instead, they often form in the middle of the apical domain (Fig. 8C,E, red arrowheads), and thus are not always associated with lateral cell junctions. This is strikingly similar to the apical positioning of wing hair primordia in *fz* or *dsh* mutants (Wong and Adler, 1993). In other cells, actin

Fig. 5. Arp3 and Ena in denticles. Embryos, anterior to top. (A-C) Deconvolved confocal images, Arp3 (red), F-actin (phalloidin; green). (A) Arp3 puncta within and surrounding actin condensations (arrowhead). (B) Arp3 enrichment as denticles elongate (arrowheads). (C) Arp3 in elongated denticles (arrowhead). (D-I) Ena (red or single channel), F-actin (phalloidin; green). (D) Denticle-producing cells accumulate cortical Ena (brackets); in other cells Ena remains at tricellular junctions (arrowheads). (E,F) Ena enriched in elongating denticles (brackets). Arrowheads, tricellular junctions in cells that do not make denticles. (G-I) Deconvolved confocal images. Blue arrowheads – Ena in tricellular junctions. (G) Actin condensations without Ena enrichment (yellow arrowhead). (H) Ena in elongating denticles (yellow arrowhead). (I) Ena in elongated denticles (yellow arrowhead). Inset, elongated denticle in cross section – Ena in denticle and at its base (white arrowhead). Scale bars, 5 μm.

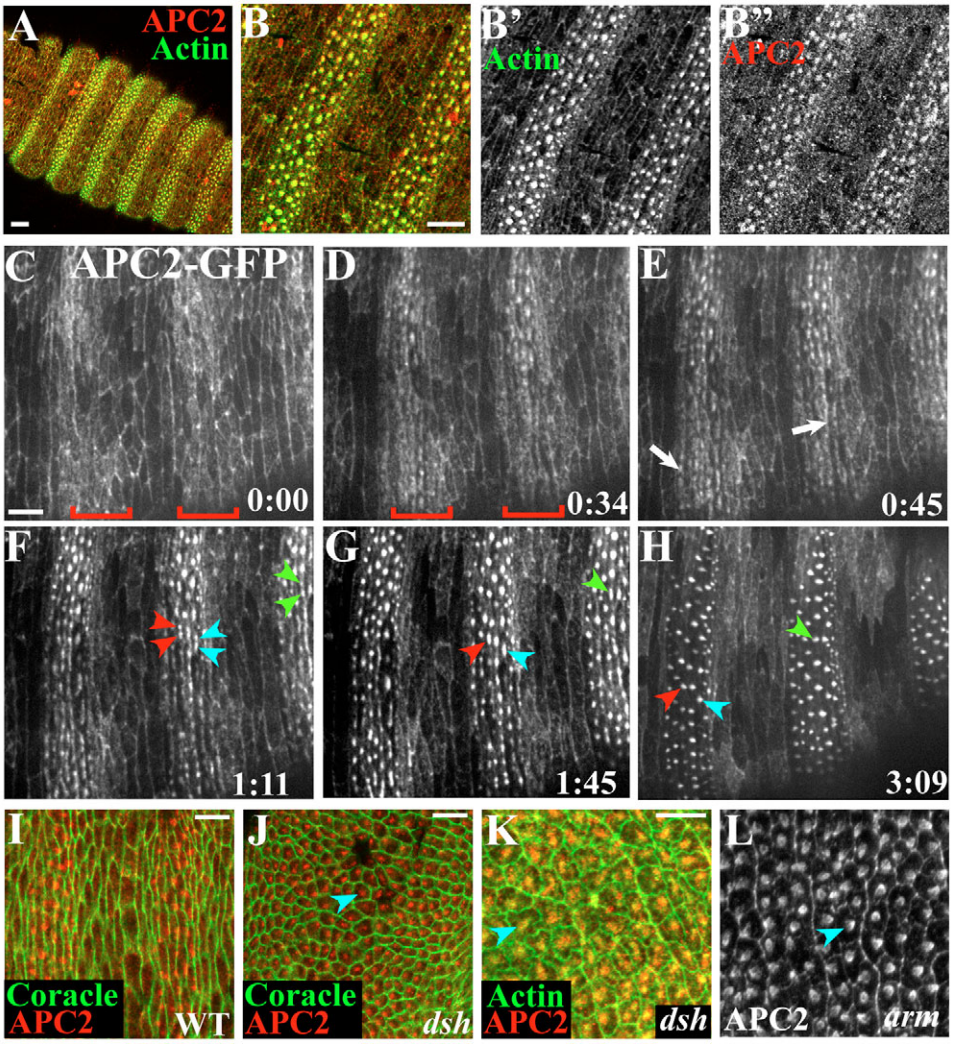


Fig. 6. APC2 co-localizes with actin throughout denticle development. Embryos, anterior left. (A,B) APC2 (red), actin (green). Late condensations. (C-H) Stills, movie of embryo expressing UAS-APC2-GFP driven throughout the epidermis using 69B-GAL4. Times in hours:minutes. (C,D) APC2-GFP localizes apically in cells of presumptive denticle belts (brackets). (E-H) APC2-GFP localizes to posterior margin (E, arrows), and condensations focus and brighten (F-H). Colored arrowheads – condensations merging. (I-L) APC2. Wild type (I) and dsh^{75} (J,K) or arm^{XM19} (L) maternal and zygotic mutants. APC2 (red, single channel in L) localizes to denticle primordia even when they arise at the cell apex (arrowheads, J-L). (I,J) Coracle (green; outlining cells). (K) F-actin (phalloidin; green). Scale bars, 10 μm.

condensations form near cell junctions, but these occur at both anterior (Fig. 8C,E, yellow arrowheads) and posterior (Fig. 8C,E white arrowheads) margins. As denticles elongate in *wg* mutants, the mirror-image nature of the final denticle pattern becomes evident, with both late condensations and denticles pointing posterior from some regions (Fig. 8E,F white arrowheads) and anterior from others (Fig. 8E,F yellow arrowheads); other denticles point toward the ventral mid-line (Fig. 8F, red arrowhead). To rule out that ectopic cell death observed in *wg* mutants lead to polarity changes, we examined embryos double-mutant for *wg* and *Df(3L)H99*, a deletion removing the three key death effectors Hid, Grim and Reaper. This blocks apoptosis, increasing cell number (each cell is smaller). All cells secrete denticles (Cox et al., 2000), but denticle polarity is disrupted as in *wg* single mutants (Fig. 8D). We also filmed live *wg* mutants expressing Moesin-GFP (Fig. 8I) (see also supplementary material Movie 4). This picture largely paralleled that in fixed tissue, with apical actin accumulation in all cells, (Fig. 8I, 1:45) and the formation of actin condensations in positions other than the posterior margin (Fig. 8I, blue and red arrows). Live imaging revealed that actin condensation formation differed in its timing. After a long period in which vague actin structures were transiently observed, well-focused condensations appeared quite quickly (Fig. 8I, 1:45-2:18, versus nearly 2 hours in wild type). Thus Wg signaling is essential for correct establishment of denticle polarity.

We next examined whether downstream components of the Wg pathway are required. Embryos maternally and zygotically mutant for dsh^{75} (null for Wg signaling) are phenotypically similar to *wg* – condensations form apically in many cells, rather than at the posterior margin (Fig. 8G, arrowheads). We also examined embryos maternally and zygotically mutant for arm^{XM19}, which retains function in adhesion but is nearly null in Wg signaling (Cox et al., 1999a; Cox et al., 1999b). Arm, and by extension the canonical Wnt pathway, do not directly regulate PCP in eye discs (Wehrli and Tomlinson, 1998). In embryos, however, the *arm* phenotype is very similar to those of *wg* and *dsh* (Fig. 8H, red arrowheads). APC2, which co-localizes with actin during wild-type denticle formation, is also mislocalized in dsh^{75} and arm^{XM19} maternal and zygotic mutants (Fig. 6J-L), localizing to apical condensations like actin.

We next examined embryos in which Wg signaling is reduced but not eliminated (arm^{XP33}zygotic mutants, retaining maternally-contributed Arm) (Peifer et al., 1991). In arm^{XP33}, all surviving cells secrete denticles, but, in contrast to *wg*,

many actin condensations have correct polarity (Fig. 8J,K, white arrows). However, in certain regions of the segment, polarity is reversed (Fig. 8J,K yellow arrows), and cells at the boundary between these two regions develop apolar condensations (Fig. 8J,K, blue arrows). Polarity reversal is maintained as denticles extend (Fig. 8L). Reducing Wg signaling by expressing a GPI-linked version of the Fz2 extracellular domain, which acts as a dominant-negative receptor (Cadigan et al., 1998), had similar effects (Fig. 8M), although in weakly affected embryos not all cells make denticles and those that do are correctly polarized (data not shown). We sought to complement these studies by examining mutants in which Wg signaling is inappropriately activated (e.g. *zw3* or *APC2*), but uniform activation of Wg signaling results in such a severe reduction in the number of denticle-producing cells that we could not draw conclusions about denticle polarity.

Determining whether Wg directly regulates denticle PCP is complicated by the fact that Hedgehog (Hh) signaling also regulates segment polarity (Nüsslein-Volhard and Wieschaus, 1980). Further, Wg and Hh are mutually dependent, with loss-of-function mutations in one pathway leading to loss of expression of the other ligand (Hidalgo, 1991). To determine Hh's role, we examined embryos mutant for the strong allele hh^{AC}. As in *wg* mutants, normal cell shape changes fail to occur, and actin condensations lose their normal polarity (Fig. 8N, white arrows). *hh* mutants also had many cells with more than one actin condensation (Fig. 8N, blue arrows). As denticles extend, most take up positions close to a cell junction (Fig. 8O). However, unlike *wg* mutants, there is no apparent segmental periodicity to the direction in which denticles point, although adjacent denticles form swirling patterns (Fig. 8O), as is seen in the ultimate cuticle pattern (Bejsovec and Wieschaus, 1993).

We also examined mutants in which the Hh pathway is activated in all cells, as this does not lead to loss of all denticles. To do so, we examined embryos mutant for *patched* (*ptc*), which inhibits Hh signaling (reviewed in Lum and Beachy, 2004). *ptc* mutants secrete denticle belts with mirror-image polarity, separated by regions of naked cuticle (Nüsslein-Volhard et al., 1984). In ptc^{9} mutants, the polarity of denticle initiation was lost, with denticles often

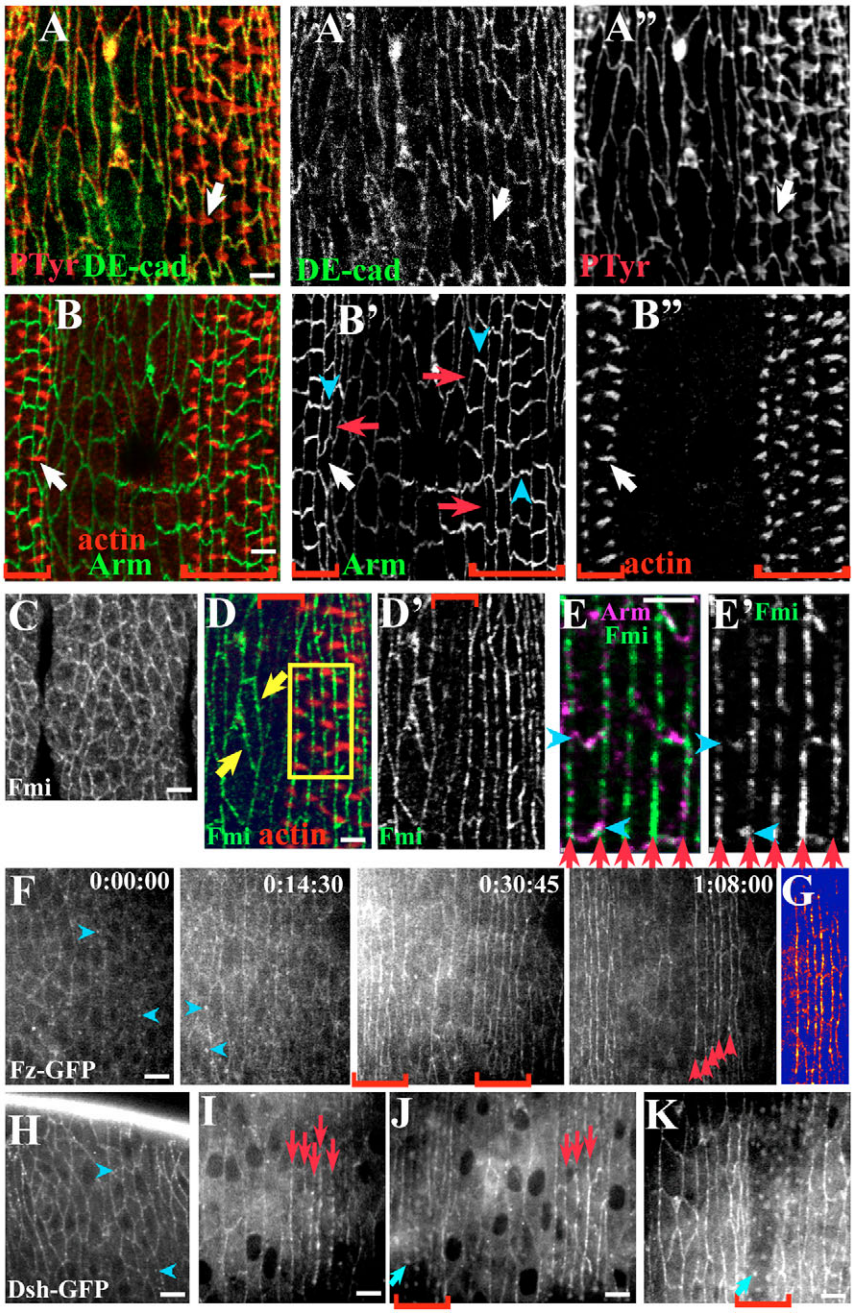


Fig. 7. Asymmetric localization of polarity proteins. Embryos, anterior left. (A) DE-cadherin (green), P-Tyr (red). (B) Arm (green), F-actin (red). (A,B) Neither DE-cadherin nor Arm localize to denticles (white arrows). Cortical Arm levels are higher in denticle-producing cells (brackets). Arm levels are somewhat higher at dorsal/ventral cell borders (blue arrowheads) than at anterior/posterior cell borders (red arrows). (C-E) Fmi (single channel or green). (C) Prior to denticle development Fmi localizes uniformly to cortex. (D,E) F-actin (red), Fmi (green), Arm (violet). Cells just anterior to denticle belts accumulate less Fmi (bracket). Cells just posterior to denticle belts accumulate Fmi uniformly on all cell interfaces (arrows). (E) Close-up, yellow-boxed region in D. Denticle-secreting cells accumulate more Fmi at anterior/posterior margins (red arrows) than on dorsal/ventral margins (blue arrowheads). (F) Stills, movie of Fz-GFP. Times in hours:minutes:seconds. (G) False-color closeup of F; pixel intensity color-coded from blue to yellow. (H-K) Still images, Dsh-GFP. Red arrows – asymmetric accumulation at anterior/posterior boundaries. (F-K) Blue arrowheads=presumptive vesicles, red arrows/arrowheads=asymmetric accumulation along anterior-posterior boundaries, red brackets=presumptive denticle belts, blue arrows=presumed actin condensations. Scale bars, 5 μm.

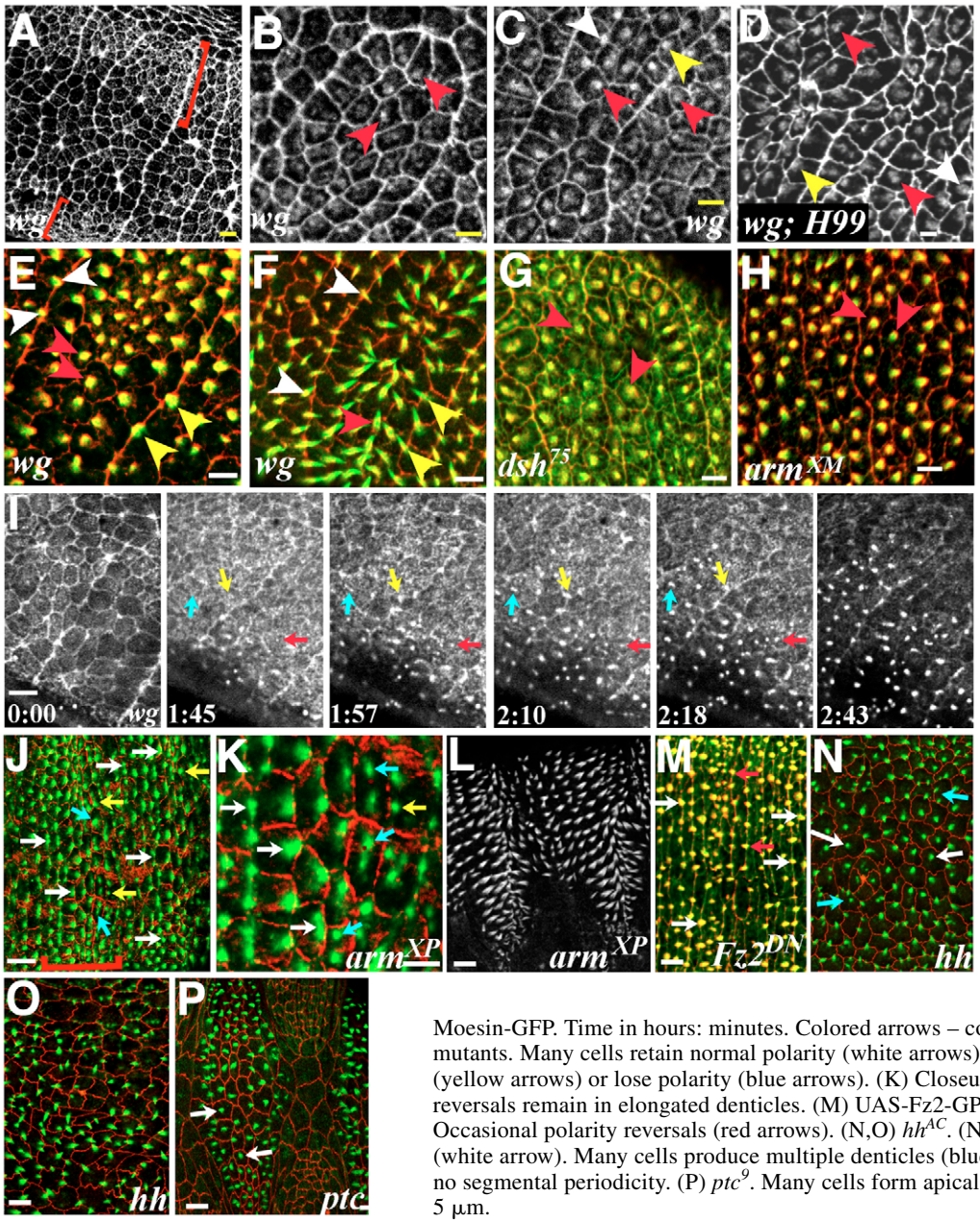


Fig. 8. Canonical Wg and Hh signaling are required for denticle polarity. Embryos, anterior left. F-actin (green or single label; phalloidin, A-H,L-M; anti-actin, J,K,N-P). Cell outlines (red; P-Tyr, E-H,M; anti-Arm, J,K,N-P). B-H. Actin condensation and denticle formation are not restricted to the posterior margin. Some denticles form at the cell apex (red arrowheads), while others form at anterior or posterior cell junctions (yellow and white arrowheads, respectively). A-C,E,F, wg^{IG22}. D, *wg; Df(3L)H99*. (A) All cells remain cuboidal and accumulate apical actin (beginning laterally; brackets). (B,C) Actin condensations form (B) and sharpen (C). (D) Denticle polarity is lost even if cell death is blocked. (E) Denticle elongation. (F) Elongated denticles. (G,H) Embryos maternally and zygotically dsh^{75} (G) or arm^{XM19} (H). (I) Stills, movie of wg^{IG22} embryo expressing Moesin-GFP. Time in hours: minutes. Colored arrows – condensations. (J-L) arm^{XP33} zygotic mutants. Many cells retain normal polarity (white arrows). A subset have reversed polarity (yellow arrows) or lose polarity (blue arrows). (K) Closeup, bracketed area in J. (L) Polarity reversals remain in elongated denticles. (M) UAS-Fz2-GPI expressed using Rho-GAL4. Occasional polarity reversals (red arrows). (N,O) hh^{AC}. (N) Condensation polarity is lost (white arrow). Many cells produce multiple denticles (blue arrows). (O) Denticles elongating; no segmental periodicity. (P) ptc^{9}. Many cells form apical condensations (arrows). Scale bars, 5 μm.

forming at the cell apex (Fig. 8P; in the cuticle a mirror-image pattern is observed, however). Thus both loss of Hh signaling and its uniform activation disrupt normal PCP.

The role of PCP proteins

We next investigated the roles of proteins specifically required for PCP. We first examined embryos homozygous mutant for a null allele of *stbm* ($stbm^{6}$ has a two-base pair deletion and thus a frame shift at amino acid 81); they are viable and thus we examined complete loss-of-function. In *stbm* mutants, certain features of denticle development are normal. Cells choose denticle and naked cuticle fates normally, normal cell shape changes occur, and there is a strong bias toward denticles initiating at posterior cell margins (Fig. 9C, blue arrowheads). However, in contrast to wild type, there are frequent defects in the placement of denticle primordia in rows 1 and 2 (e.g. Fig. 9C, white arrowheads). These often form in the center of the cell or at the anterior margin. We observed similar defects in embryos zygotically mutant for *fmi* (Fig. 9D,E white arrowheads; *fmi* mutations are lethal; zygotic mutants were identified using a GFP-marked Balancer). We also examined embryos in which maternal Dsh was eliminated, and zygotic Dsh was encoded by the PCP-specific mutant dsh^{1}. These embryos also had defects in denticle polarity in rows 1 and 2 (Fig. 9F, data not shown), although these were not as penetrant as those of *stbm* or *fmi*. However, dsh^{1} may not be fully null for PCP function. Finally, we examined embryos maternal and zygotically mutant for pk^{1}. Consistent with earlier observations of cuticles (Gubb et al., 1999), we saw few defects in denticle polarity (Fig. 9G).

The two *Drosophila* Wg receptors, Fz and Fz2, both transduce canonical Wnt signals but Fz plays a more

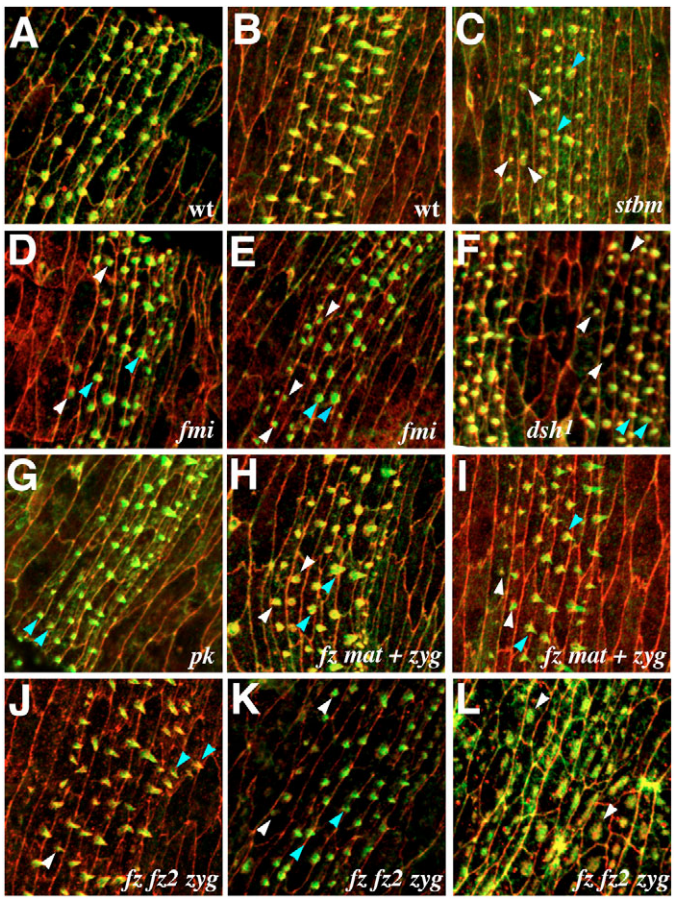


Fig. 9. Roles for PCP proteins.. Embryos, anterior upper left. F-actin (green; phalloidin). Cell outlines (red; P-Tyr). White arrowheads=mispositioned denticles, blue arrowheads are correctly-positioned denticles. (A,B) Wild type, (C) Maternal and zygotic *stbm*^{6}. (D-E) Zygotic *fmi*^{192}. (F) Maternally *dsh*^{75}, zygotically *dsh*^{1}. (G) Maternal and zygotic *pk*^{pk1}. (H,I) Maternal and zygotic *fz*^{D21}/*fz*^{R52}. (J-L) Zygotic *fz*^{D21} *Df(3L)Dfz2*/*fz*^{R52} *Df(3L)Dfz2*.

important role in PCP. We thus investigated their roles in denticles. Embryos maternally and zygotically mutant for strong/null *fz* alleles had defects similar to or stronger than those of *stbm* or *fmi* mutants (Fig. 9H,I); once again, however, defects were largely confined to denticle rows 1 and 2. If one removes Fz and Fz2 maternally and zygotically, embryos have a strong phenotype similar to complete loss of Wg signaling (Bhanot et al., 1999). To probe whether Fz2 also plays a role in polarity, we reduced function less dramatically, removing both Fz and Fz2 zygotically but not maternally. These embryos have phenotypes ranging from wild type to very mild to moderate segment polarity defects (Bhanot et al., 1999). We observed defects in denticle polarity consistent with this phenotypic range. In some cases, we observed small numbers of ectopic denticles, but both ectopic and normal denticles had correct polarity (Fig. 9J). In other cases, we observed mild defects in polarity similar to those of *stbm* or *fmi* (Fig. 9K). Finally, in occasional embryos, we saw more striking effects on naked cuticle specification (Fig. 9L), with effects on polarity more similar to those seen of zygotic *arm* mutants.

Discussion

PCP is a widespread property of animal epithelia. Polarization requires two steps: cells must interpret directional cues along the relevant body axis, and translate these cues into cytoskeletal polarity. The *Drosophila* wing is the best-studied model of this process, and many protein players involved in both aspects of PCP have been identified there. However, the connections between signal perception and cytoskeletal responses are not well understood. Further comparisons of wing and eye imaginal discs revealed that PCP has similarities and differences in different tissues. Additional examples of PCP thus reveal the range of cellular mechanisms governing polarity establishment, and identify those that are conserved and those that diverge. In addition, live cell imaging may reveal aspects of cytoskeletal rearrangement that are not obvious in fixed tissue, so the ideal system would allow easy visualization. The embryonic denticles provide such a system.

The cytoskeletal events underlying polarity establishment

Among the hallmarks of PCP in structures as diverse as *Drosophila* wing hairs to stereocilia in the mammalian ear is polarization of the actin cytoskeleton. Pioneering work by Paul Adler's lab revealed the polarized actin cytoskeleton underlying wing hair polarity and documented defects in polarization in *fz* and *dsh* mutants (Wong and Adler, 1993). MTs are also polarized in developing wing hairs (Eaton et al., 1996; Turner and Adler, 1998), and disruption of either actin or MTs disrupts wing hair formation (Turner and Adler, 1998). Our data suggest that basic features of cytoskeletal polarity in pupal wing hairs are also seen in denticles. Denticles, like wing hairs, arise from polarized actin accumulations – in denticles this occurs along the posterior cell margin. Further, like wing hairs, denticles all elongate in the same direction. Our less detailed analysis of dorsal hairs suggests that they also arise from polarized actin accumulations, but these are more complex, as different cell rows accumulate actin either along the anterior or posterior cell margin.

The effect of Wg and Hh on denticle development is mediated in part by their regional activation of the Shaven-baby transcription factor, which is necessary and sufficient for cells to generate actin-based denticles (Payre et al., 1999). Therefore genes that are targets of Shaven-baby are likely to be triggers for actin accumulation and cytoskeletal rearrangements. Wg and Hh signaling may also trigger polarization of cellular machinery that is not typically thought to be involved in PCP – e.g. the polarity of Arm we observed. It will be useful in the future to examine whether proteins polarized during germband extension, such as Bazooka (reviewed in Pilot and Lecuit, 2005), are also polarized during denticle formation. Mutations in both *hh* and *wg* also affected the normal changes in cell shape accompanying denticle formation – rather than elongating along the dorsal-ventral axis, cells remain columnar. We observed a similar failure of cells to polarize during dorsal closure in *wg* mutants (McEwen et al., 2000). These effects may reflect alterations in cell polarization or cytoskeletal regulation. It will be of interest to determine whether changes in cell shape are coupled to the establishment of cytoskeletal polarity.

Thus far the analysis of actin in wild-type and mutant pupal wings was restricted to snapshots in fixed tissue. We extended

this by examining F-actin in developing denticles in real time, revealing features of polarization that were not previously noted, which may be shared with wing hairs or other polarized structures. The initial cytoskeletal change we observed was actin accumulation all across the apical surface of the cell. This actin gradually 'condenses', becoming more restricted to the posterior cell margin and forming distinct condensations, which then brighten and sometimes merge. They then elongate, all in the posterior direction. It will be interesting to learn whether the dynamic aspects of condensation involve de novo actin polymerization and/or collection of preexisting actin filaments.

It is only in late condensations that we saw enrichment of any of the actin regulators we examined. Arp3 and Dia are weakly enriched in late condensations, with enrichment increasing as denticles elongate, and Ena is enriched even later. Of course, the localization of these actin regulators to developing denticles does not by itself demonstrate that they play an important role there, but it is consistent with the possibility that they have a role in actin remodeling associated with denticle elongation. To test this hypothesis, genetic analyses will be necessary. This presents significant obstacles, as Arp2/3 (Stevenson et al., 2002) and Dia (Afshar et al., 2000) are required for much earlier events (syncytial stages and cellularization), while maternal Ena plays a role in oogenesis (J. Gates, J. P. Mahaffey and M.P., unpublished data), complicating analysis of loss-of-function mutants. Surprisingly, none of these actin regulators localizes in an informative fashion during the initial formation of actin condensations (though APC2 localizes there during this time). Thus we need to identify additional regulators functioning during early denticle development. Studies of cytoskeletal regulation in the larger adult sensory bristles may guide this. EM studies, the use of cytoskeletal inhibitors, and FRAP, which proved informative in studies of wing hairs and bristles (Fei et al., 2002; Tilney et al., 1995; Tilney et al., 1996; Tilney et al., 2000a; Turner and Adler, 1998), may reveal how actin in denticles is assembled. Finally, it will be important to study in denticles additional actin regulators that regulate bristle development (Hopmann and Miller, 2003; Wahlstrom et al., 2001).

What signals regulate denticle polarity?

As examples of PCP have proliferated, our understanding of the signals that instruct cells about their orientation in epithelial sheets has evolved. Certain features are shared in many, if not all, tissues. Fz receptors play a key role. Other core polarity proteins including Dsh, Fmi, Van Gogh/Strabismus and Prickle act in many if not all places. Our data extend this analysis to the denticles. We found intriguing differences between the phenotypes of loss of Wg or Hh signaling, in which polarity was severely altered or abolished and loss of proteins that play dedicated roles in PCP, such as embryos null for either *fz* or *stbm*, which exhibited more subtle defects. A strong polarity bias was retained in these latter mutants, with cells in the posterior denticle rows correctly polarized and only cells in the anterior two rows making frequent mistakes. Interestingly, occasional mistakes are also observed in wild-type embryos (albeit at much lower frequency) and these are also restricted to the anterior most rows. This is in strong contrast to the effects of these mutants in the wing disc, where they globally disrupt polarity.

One possible reason for this difference is the different scales of the tissues. The embryonic segment is only 12 cells across, while the wing disc encompasses hundreds of cells. Many core polarity proteins help mediate a feedback loop that amplifies an initially small difference in signal strength between the two sides of a wing cell. Perhaps the small scale of the embryonic segment makes this reinforcement less essential. It is also intriguing that the polarity is most sensitive to disruption in the anterior two denticle rows. If signal emanated from the posterior, signal strength might be lower in the anteriormost cells, rendering the reinforcement process more important. The lower frequency of defects in pk^1 mutants may also reflect the reduced role of the feedback loop, but this is subject to the caveat that *pk* is a complex locus with different mutations having different consequences (Gubb et al., 1999). Future work will be needed to test these possibilities.

Significant questions also remain about the signal(s) activating Fz receptors during PCP. Wnts were initial candidates, as Fz proteins are Wnt receptors. In vertebrates, this may be the case – Wnt11 regulates convergent extension (Heisenberg et al., 2000) and Wnt proteins can regulate PCP in the inner ear (Dabdoub et al., 2003). By contrast, *Drosophila* Wnt proteins may not play a direct role. The Wg expression pattern in the eye and wing discs is not consistent with a role as the PCP ligand. Detailed studies of PCP in the eye and abdomen are most consistent with the idea that neither Wg nor other Wnt proteins are polarizing signals, but suggest that Wg regulates production of a secondary signal [dubbed 'X' by Wehrli and Tomlinson (Wehrli and Tomlinson, 1998) and Lawrence et al. (Lawrence et al., 2002)]. Recent work suggests that Fj, Ds and Fat may be this elusive signal (see Introduction), with *Drosophila* Wg acting as an indirect cue of polarity. In fact, one cannot rule out the possibility Wnt11's role in vertebrate convergent extension is also indirect.

We found roles for Wg, Dsh and Arm in establishing denticle polarity. At face value, Arm's role is surprising, as the current view is that the Wg pathway diverges at Dsh, with a non-canonical branch mediating PCP and the canonical pathway playing no role in this. However, our data do not imply that Arm is required in denticle PCP per se. Wg acts in a paracrine feedback loop to maintain its own expression (Hidalgo, 1991; Martinez-Arias et al., 1988). In embryos maternally and zygotically mutant for *arm* alleles that cannot transduce Wg, Wg expression is lost by late stage 9 (Peifer et al., 1991). Thus, even though Arm is not in the non-canonical pathway, loss of Arm could still disrupt PCP indirectly due to the loss of Wg expression.

While our data demonstrate that Wg is required for denticle PCP, two things suggest its role is indirect. *wg* mutants retain segmental periodicity in denticle orientation, suggesting that polarity is not totally disrupted, while in *hh* mutants there is no segmental periodicity. Second, when we reduced but did not eliminate Wg signaling, many cells retained normal polarity and there was segmental periodicity to which cells lost polarity or exhibited polarity reversals. This is consistent with the idea that Wg regulates production of another ligand. In fact, Wg's role may be even more indirect – given the more dramatic effect of *hh*, Wg's primary role in polarity may be to maintain Hh expression (this is also consistent with a requirement for canonical pathway components like Arm). Global activation of Hh signaling in the *ptc* mutant also disrupts polarity. Hh thus

remains a possible directional cue. In the abdomen, Hh also plays an important role in polarity, but it does not seem to be the directional cue either but rather regulates its production; this may also be the case in the embryo. Thus the precise roles for canonical Wg and Hh signaling in denticle polarization must be addressed by future experiments. If neither Wnts nor Hh are directional signals, what is? Data from the eye, wing and abdomen suggest roles for Ds, Fj, Fat and Fmi but details differ in different tissues. It thus will also be useful to examine Ds, Fj and Fat's roles in embryonic PCP.

Materials and Methods

Fly strains

Experiments were done at 25°C. FlyBase describes alleles used (flybase.bio.indiana.edu). Wild type was *yellow white* or Canton-S. *arm* and *dsh* maternal and zygotic mutants were made as in Cox et al. (Cox et al., 1996). UAS-Moesin::GFP (Edwards et al., 1997) was from D. Kiehart (Duke University), UAS-APC2::GFP (Cliffe et al., 2004) from M. Bienz, (MRC, Cambridge, UK), UAS-GPI::Fz2 (Cadigan et al., 1998) from K. Cadigan (University of Michigan), arm-Fz::Gfp (Strutt, 2001) from D. Strutt (University of Sheffield) and dsh-Dsh:GFP (Axelrod, 2001) from J. Axelrod (Stanford University).

Time-lapse microscopy

Bleach-dechorionated embryos were mounted in halocarbon oil (series 700; Halocarbon Products Corporation) between a coverslip and a gas-permeable membrane (petriPERM; Sartorius Corporation). Images were captured using an Ultraview Confocal Microscope (PerkinElmer). Image analysis was performed with Ultraview Software, NIH Image, or MetaMorph (Universal Imaging).

Immunolocalization

Bleach-dechorionated embryos were fixed in 1:1 heptane:37% formaldehyde, for 5 minutes and methanol-devitellinized unless noted. Variations: Phalloidin, hand-devitellinized. Anti-actin, heat-fixed (Peifer, 1993). Anti-β-tubulin+phalloidin, 10:9:1 heptane:37% formaldehyde:0.5 M EGTA, 6 minutes, hand-devitellinized. Anti-Fmi, 8% paraformaldehyde+20 mM $CaCl_2$ 30 minutes. Anti-DE-cadherin, 4% formaldehyde, 20 minutes. All were blocked, washed and stained in PBS/1% goat serum/0.1% TritonX-100, except anti-Fmi, for which PBS/0.5%BSA/0.3%TritonX-100 was used. Primary antibody incubations were overnight at 4°C; phalloidin and secondary antibody incubations were 2-3 hours at room temperature; embryos stained with phalloidin alone for deconvolution were incubated overnight at 4°C. Primary antibodies: mouse monoclonals: anti-phosphotyrosine (Upstate Biotechnology), 1:1,000; anti-β-tubulin, 1:5000; anti-Ena (both DSHB), 1:500; anti-actin, 1:500 (Chemicon International, Inc.); anti-Fmi (T. Uemura), 1:10; anti-Arm, 1:500; anti-Coracle16B+9C (R. Fehon), 1:500; rat polyclonal anti-APC2, 1:1,000 (McCartney et al., 1999); rat monoclonal anti-DE-cadherin (T. Uemura), 1:200; rabbit polyclonals: anti-Dia (S. Wasserman), 1:500; anti-Arp3 (W. Theurkauf), 1:200. Secondary antibodies were Alexa 488, 568 and 647; actin was visualized using Alexa 488, 568 and 647 phalloidin (Molecular Probes, Inc.). Embryos were mounted in Aqua Polymount (Polysciences, Inc.) and imaged with LSM410 or 510 confocal microscopes (Carl Zeiss MicroImaging, Inc.). Image deconvolution was performed on confocal stacks using a softWoRx Imaging Workstation (Applied Precision). Images were prepared and contrast and brightness adjusted using Adobe Photoshop. To prevent artificially increasing resolution when images were enlarged, no interpolation was done.

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